

The University of Chicago

PIGMENTATION STUDIES
IN SALAMANDERS, WITH ESPECIAL
REFERENCE TO THE CHANGES
AT METAMORPHOSIS

A DISSERTATION SUBMITTED TO THE FACULTY
OF THE DIVISION OF THE BIOLOGICAL SCIENCES
IN CANDIDACY FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY

DEPARTMENT OF ZOÖLOGY
MARCH, 1946

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PIGMENTATION STUDIES IN SALAMANDERS, WITH ESPECIAL REFERENCE TO THE CHANGES AT METAMORPHOSIS¹

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ALTHOUGH the amphibian pigmentary pattern changes conspicuously at metamorphosis, few investigators have attempted to describe the developmental processes which take place. Most of the literature contains only brief descriptions of the gross changes which are visible externally (Banta and McAtee, 1906; Uhlenhuth, 1916, 1917; Grant, 1930). Those who have studied the histological aspects of the problem have described only the typical embryo, larva, and adult and have made no attempt to show the sequence of events which leads to the definitive pigmentary pattern and the complete development of the dermis (Dawson, 1920; Wilder, 1925; Berweger, 1926; Elias, 1931, 1934). Three types of pigment cells are commonly found in the larval and adult stages of amphibians and have been noted by many authors (Weidenreich, 1912; Fuchs, 1914; Biedermann, 1926, 1928, 1930). They are (1) the lipophores or xanthophores, which contain a red or yellow pigment; (2) the guanophores, which contain highly refractile white or yellow crystals; and (3) the melanophores, which contain dark-brown or black pigment granules. The present investigation is primarily concerned with

the melanophores which occur in the skin, although this type of pigment cell is widely distributed in the body of amphibians.

The theories which have been advanced concerning the origin of vertebrate pigment cells have been reviewed by Fuchs (1914) and Biedermann (1926, 1928, 1930) and, more recently, by DuShane (1943, 1944). At present the origin of melanophores from the neural crest in amphibians and birds has been so well established that other hypotheses no longer need to be considered. Until the last decade it was generally accepted that the dermis was derived from the mesoderm. More recently some investigators have suggested that the neural crest plays at least some part in dermal development (Raven, 1931, 1936; Holtfreter, 1935; Detwiler, 1937, 1938). Their evidence is largely inconclusive, however, because the work has been confined, for the most part, to early developmental stages when the dermis is poorly differentiated.

The object of the present investigation is to describe the histological development of pigmentation in the metamorphosing urodele and to correlate this with the gross pigmentary changes which occur at this time. A series of animals from the just-hatched larva to the adult was included in the study when it became apparent that many of the important pigmentary changes occur in the larva. However, it was soon realized that the development of the pigmentary pat-

¹ This paper is a revision of a dissertation presented to the faculty of the University of Chicago in partial fulfilment of the requirements for the degree of Doctor of Philosophy. I wish to express my sincere gratitude to Professor G. P. DuShane for suggesting this problem and for his aid during the investigation.

tern could not be described without considering the history of the dermis. Xenoplastic neural-crest grafts were used to aid in the analysis of some of the factors which influence the pigmentary pattern. This method is of value in determining what part of the specific differences in this pattern is controlled by the host environment and what part by factors intrinsic to the cells themselves. The graft material has been of special value in determining the relation between epidermal melanophores and the melanin found in epidermal cells.

MATERIALS AND METHODS

The animals used in this investigation are *Ambystoma tigrinum*, *Ambystoma maculatum*, and *Triturus torosus*. The specimens of *A. tigrinum* and *A. maculatum* were obtained from eggs collected in ponds in the Chicago region. Specimens of *T. torosus* were obtained from eggs shipped from Stanford University, through the courtesy of Professor V. C. Twitty. After hatching, the larvae were isolated in finger bowls and kept in well water, which was changed every 3 or 4 days. For 2 or 3 weeks the larvae were constantly supplied with white annelid worms of the genus *Enchytraea* to assure maximum feeding. Thereafter, the larvae and, later, the adults were fed once a day with strips of raw liver.

In all cases length was determined by measurement from snout to tip of tail. The entire animal was then fixed in formol-Zenker's solution for 24 hours. In the normal series the head was cut off behind the neck in the smaller animals, while in the larger ones a square of skin and underlying muscle was removed from the dorsal cervical region. These pieces were imbedded in paraffin, while the rest of the animal was stored in 70 per cent alcohol.

The neural-crest grafts, which were

made by Professor DuShane, consisted of grafting pieces of trunk neural folds orthotopically between embryos of *A. maculatum* and *T. torosus* of stages 14-22. In the graft series the anterior half of the animal was imbedded, and sections were made in the region of the graft. All sections were cut at 10μ and stained either in Heidenhain's modification of Mallory's connective-tissue stain or in hematoxylin eosin azur.

In order to determine whether the increase in cell number observed at certain periods of development was the result of increased mitoses or of cell migration from other regions, two colchicine series were set up. Larvae of *A. tigrinum* were placed in a solution of 20 mg. colchicine per 100 cc. well water for 29 hours, while larvae of *A. maculatum* were placed in a solution of 100 mg. per 100 cc. well water for the same length of time. These were the approximate concentrations used by Mills (1939) in her work on *A. jeffersonianum*. The mitotic index (number of mitoses per 100 cells) was obtained from a count of at least one thousand cells of each type.

To aid in analyzing changes in the cell types which may be important in the development of pigmentation and of the dermis, counts were made of a standard area (four $10\text{-}\mu$ sections, three fields per section, with an 8-mm. objective). To reduce variation the counts were made in a corresponding region in all animals: the mid-dorsal area of the skin in the cervical region. To obtain a representative sample, every eighth section was counted. It is obvious that there is an inherent disadvantage in this method: the surface of the animal increases manyfold, so that in the just-hatched larva the area counted represents a much larger portion of the total body surface than does an area of the same size in an adult. Since the area counted is small, the

sampling error may be large. Melanophores are difficult to count accurately because the nuclei are often obscured by the melanin granules.

Measurements of dermal thickness were obtained by means of an ocular micrometer. Each value given is the average of six measurements made over as much of the dorsal surface as the section covered. There is considerable variation in different regions; hence the values are only approximations. They are considered significant, however, since the increase in thickness which takes place during development is a very large one.

DEVELOPMENT OF PIGMENTATION AND THE RELATED DEVELOPMENT OF THE DERMIS

The arrangement of melanophores in the skin varies greatly among different species of urodeles. The following observations were made on three species representing two different families. Although basic similarities will be brought out, it will become apparent that many small differences are present, especially between different families.

NORMAL DEVELOPMENT

A series of animals from the young larva through metamorphosis to the adult stage was obtained for each of the three species studied. For *A. tigrinum*, the interval between each animal in the series is about 5 mm., while in *A. maculatum* and *T. torosus*, which are smaller and slower developing, the interval is about 3 mm. Although it is the metamorphic changes with which this investigation is most concerned, it is necessary to recognize the earlier changes during the larval period in order to appreciate that the metamorphic changes are only the climax of earlier development. In *T. torosus*, study was also made of the first

appearance of melanophores in the embryo.

A. tigrinum AND *A. maculatum*

The sequence of events in the development of the dermis and of the pigmentary pattern is very similar in these species, with only minor variations. Plate I, showing the development of the skin of *A. tigrinum*, can serve to indicate the development in *A. maculatum* as well. In the larva which has just hatched, melanophores are found in the loose connective tissue of the subdermal region and in the epidermis. Subdermal melanophores are closely applied to the overlying layer of collagenous fibers which comprises the dermis at this stage (see *A*, Pl. I). This layer of fibers is extremely thin—2–4 μ in thickness—and contains no cells. In addition to melanophores, stellate-shaped cells containing no pigment also occur in the subdermal region, their processes adherent to the overlying dermis. There is no morphological basis for distinguishing these cells from the undifferentiated mesenchyme cells of the deeper loose connective tissue. It will be shown later, however, that the mesenchyme-like cells immediately below the dermis appear to contribute to the formation of the dermis and that at least a part of them are potential pigment cells. For this reason these cells will be referred to as “unpigmented cells.” This does not mean that all cells referred to as unpigmented will become chromatophores, for this is certainly not the case. It is used for want of a better term to indicate that these cells, because of their position, may have a different prospective potency than the cells which occur at random in the loose connective tissue. In making the counts, only those cells were included which were located adjacent to the dermis.

There is no significant increase in the

number of subdermal melanophores per unit area during the first half of the larval period (Table I, *A. tigrinum*, and Table 2, *A. maculatum*). There must be an increase in the total number, however, because the surface area has increased several fold during this time. The

the proportion of rounded cells increases so that very few expanded cells are found directly below the dermis by the mid-larval period. The increase in cell number can be correlated with an increase in the mitotic index (number of mitoses per 100 cells), which is highest during the

TABLE 1
RELATION OF THE NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT *Ambystoma tigrinum*

LENGTH (MM.)	Description	SUBDERMIS			DERMIS				EPI- DER- MIS	TOTAL	
		Unpigmented Cells		Mel- ano- phores	Unpigmented Cells		Mel- ano- phores	Thick- ness (μ)	Mel- ano- phores	Unpig- ment- ed Cells	Mel- ano- phores
		Num- ber	Mi- to- tic Index		Num- ber	Mi- to- tic Index					
24.....	Larva	47	6.3	17	0	0	0	3	8	47	25
29.....	Larva	123	6.7	13	0	0	0	4	4	123	17
33.....	Larva	119	3.7	8	0	0	0	7	7	119	15
39.....	Larva	145	1.8	17	0	0	0	8	13	145	30
44.....	Larva	187	1.3	12	2	0	0	10	7	189	19
48.....	Larva	214	1.7	16	1	2.6	0	12	9	215	25
53.....	Larva	171	2.0	16	3	0.3	1	13	11	174	28
58.....	Larva	193	0.8	19	3	0.2	1	17	7	196	27
63.....	Larva	207		9	5		1	14	17	212	27
67.....	Larva	206	1.0	11	13	2.6	3	20	3	219	17
73.....	Larva	179		2	123		9	33	8	302	19
79.....	Larva	109	2.3	3	171	7.4	19	41	6	280	28
85.....	Larva	99	2.0	2	211	3.5	19	61	3	310	24
92.....	Larva	70		1	215		13	116	3	285	17
97.....	Metam. I	77		0	225		18	120	4	302	22
100.....	Metam. II	74		2	207		23	105	6	281	31
107.....	Metam. III	107		0	268		29	185	1	375	30
117.....	Adult I	55		9	345		29	307	5	400	43
135.....	Adult II	52		10	367		49	324	17	419	76

subdermal unpigmented cells, on the other hand, increase very rapidly until the mid-point in larval development (50 mm. in *A. tigrinum*; 33 mm. in *A. maculatum*). During this time the unpigmented cells appear stellate-shaped for the most part, with elongated processes extending for some distance below the dermis; but some are rounded in shape, with an eccentrically placed nucleus and more basophilic cytoplasm. However,

early larval period. Tables 1 and 2 give the mitotic index for dermal as well as subdermal unpigmented cells. This was obtained on animals of lengths corresponding to those in the normal series. An occasional mitosis was observed in melanophores, but the rate is too low to be measured with any degree of accuracy. While the subdermal unpigmented cells are increasing in number, the layer

of collagenous fibers, which constitutes the dermis, increases in thickness to about 12 μ . Although the subdermal unpigmented cells are, for the most part, rounded in shape at this time, one may commonly see one or two fine processes extending toward the dermis (see B, Pl.

process extending from the subdermal region (see C, Pl. I).

In *A. maculatum* the appearance of cells in the dermis is paralleled by changes in the pigmentary pattern of the living animal. The larva assumes a darker appearance quite suddenly, and

TABLE 2

RELATION OF THE NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT *Ambystoma maculatum*

LENGTH (MM.)	DESCRIPTION	SUBDERMIS			DERMIS				EPI- DER- MIS	TOTAL	
		Unpigmented Cells		Mel- ano- phores	Unpigmented Cells		Mel- ano- phores	Thick- ness (μ)	Mel- ano- phores	Unpig- ment- ed Cells	Mel- ano- phores
		Num- ber	Mi- totic Index		Num- ber	Mi- totic Index					
14.....	Larva	81	6.4	10	0	0	0	3	11	81	21
20.....	Larva	117	2.5	15	0	0	0	3	8	117	23
24.....	Larva	125	0.6	11	0	0	0	7	7	125	18
27.....	Larva	121	5.0	9	1	0	0	10	4	121	13
32.....	Larva	158		15	2		0	13	8	160	23
36.....	Larva	165	5.7	19	6	12.9	2	18	9	163	30
39.....	Larva	116	3.3	6	43	7.5	5	16	6	159	16
42.....	Larva	107		2	120		16	25	9	227	27
45.....	Larva	122		0	147		18	46	6	269	24
46.....	Larva	95	0.2	0	168	0.4	19	66	6	263	25
48.....	Metam. I	94		0	171		18	69	11	265	29
48.....	Metam. II	110		0	216		35	60	11	326	46
47.....	Adult I	76		1	162		49	68	7	238	57
48.....	Adult II	58		0	148		28	75	11	206	39
60.....	Adult III	69		2	182		61	91	5	251	68

I). Some of these processes penetrate the fibrous layer and extend laterally between the fibers. The fibers of the dermis are now more loosely arranged, and the dermis is thicker. The first appearance of entire cells within the dermis occurs soon after this, and many cells may be found with part of the cell body within the dermis and part below it. Subdermal melanophores are frequently seen with processes extending into the dermis, and rarely the major part of the cell body occurs in the dermis, with only a single

light spots appear along the flanks, indicating the position of the lateral-line organs. This change is a conspicuous one and was described by Uhlenhuth (1917) as the formation of the "network stage." No such change in external appearance has been noted in *A. tigrinum* to mark the first appearance of cells in the dermis.

In the more advanced larva there is a rapid increase in the number of melanophores and unpigmented cells in the dermis and, at the same time, a de-

crease in number of subdermal cells. This is accompanied by an increase in the thickness of the dermis and a looser arrangement of the fibers. As this degree of development is reached, a capillary net is established in the dermis for the first time. Skin glands begin to appear, first, as small buds that are downgrowths of the basal layer of the epidermis. The anlagen increase in size rapidly, and, as they push down into the dermis, they appear to push the collagenous fiber layer down before them. This results in a region of loose connective tissue which will soon make up the greatest part of the dermis and in which the cells accumulate. The increase in number of dermal unpigmented cells during this time may, in large part, be accounted for by the high mitotic index of these cells. Although it is probable that the greatest part of any further increase in number of dermal unpigmented cells is the result of mitoses, throughout the larval period and at times even in the adult one may see subdermal unpigmented cells partially imbedded in the fibrous inner layer of the dermis. The distorted shape of the nuclei of many of these cells strongly suggests that these cells are migrating from a subdermal position into the dermis.

Although the larvae become progressively darker as they approach metamorphosis, there is no corresponding increase in the number of melanophores per unit area. However, the darkening color may be correlated with the change in position of the melanophores, which now occur principally in the dermis instead of in the subdermis. This more superficial position under the epidermis may make them more visible, so that they are more conspicuous externally. There has been, however, a large increase in the number of unpigmented cells, which appear to be concerned in the develop-

ment of the dermis. The subdermal region, at this time constituting the loose connective-tissue layer under the dermis, contains only a small number of expanded unpigmented cells and an occasional melanophore.

In the advanced larva, which is soon to metamorphose, the dermis is relatively thick and loose and consists of three layers. Immediately below the epidermis there is a thin dense layer of connective-tissue fibers, that contains no cells and is interrupted only by the ducts of the skin glands. Below this, a layer of loose connective tissue forms the major part of the dermis and contains the bodies of the skin glands, now fairly well differentiated, and the dermal cells. The loose network of fibers which fills the area between the glands contains melanophores and unpigmented cells, as well as numerous capillaries. The deepest layer of the dermis consists of densely arranged connective-tissue fibers, in which are imbedded a few unpigmented cells but no melanophores and which is penetrated by the capillaries. This fibrous layer sharply delimits the dermis from the underlying regions.

The criteria of beginning metamorphosis in the living animal are arbitrary ones, but for this purpose they will be considered to be the first indication of the reduction of the gills and the dorsal fin. These changes are noted in *A. tigrinum* at about 100 mm., while in *A. maculatum* they appear at about 50 mm. There is, however, no striking change in the histological structure of the skin corresponding to the beginning of external metamorphic changes. The increase in the number of dermal melanophores which occurs during metamorphosis is begun in the advanced larva. The melanophores, dispersed throughout the loose layer of the dermis, are large cells with greatly expanded processes. In *A.*

tigrinum a gradual increase in number of dermal unpigmented cells occurs during metamorphosis, but in *A. maculatum* any significant increase in number ceases prior to the beginning of metamorphosis. The dermis becomes thicker, chiefly through further increase in the intermediate layer, although the inner layer of densely packed fibers becomes thicker also. The subdermal region is not changed from the premetamorphic condition. The skin glands increase in size during metamorphosis and complete their differentiation. Grossly, the skin acquires a smoother appearance, probably as a result of the disappearance of the Leydig cells. In *A. tigrinum* melanophores are evenly distributed over the back and flanks, so that the dorsal surface becomes uniformly black, in contrast to the yellowish-green of the young larva. In *A. maculatum*, however, small, indistinct yellow spots appear on the black ground color of the dorsal surface. These become clearer and less numerous, so that they are fairly large and distinct when metamorphosis is completed.

There is a shift in the arrangement of melanophores in the dermis at the completion of metamorphosis. In the adult that has just completed metamorphosis, melanophores are arranged in the outer part of the loose fibrous region to form a fairly complete network beneath the thin outer layer of the dermis, and only a few processes are found deeper in the dermis. The layer of melanophores is continued around the glands so that they are enveloped by a network of melanophores. In adults of about 2 months, the oldest animals in these series, the number of dermal melanophores reaches the highest values observed here. The skin glands are large and occupy the major part of the dermis, and their cells contain a large amount of elaborated secretion. The dermis is very thick ($325\ \mu$ in *A. tigri-*

num; $90\ \mu$ in *A. maculatum*) when compared to the young larva, whose dermis is only about $4\ \mu$ thick. Unpigmented cells are rarely found immediately below the dermis. Subdermal melanophores have become more numerous, perhaps significantly so, but their position appears to correspond to the presence of blood vessels and may have no relation to the pigmentation of the skin. These features are brought out in *D*, Plate I, which shows the structure of the skin of the adult *A. tigrinum*. The adult coloration of this species is a black ground color over the back, with irregularly distributed yellow or cream spots. The ventral surface is a dark gray and contains a large number of cream spots. This is the definitive pattern of this species and will persist throughout the life of the animal. The adult coloration of *A. maculatum* is an intense black on the dorsal surface and flanks and a dark gray on the ventral side. The yellow spots are now clearly outlined and form two rows along the entire length of the back.

Tables 1 and 2, previously referred to, give in detail the exact number of cells per unit area for each animal in the series. The totals of dermal and subdermal unpigmented cells are included to show the change in number of this cell type during development. In summing these cells, it is assumed, of course, that subdermal and dermal unpigmented cells are related, a conclusion suggested by the correspondence in time between the decrease in number of subdermal unpigmented cells and the first appearance and subsequent increase in dermal cells of the same type. Further evidence in support of this hypothesis is the frequent appearance at this time of subdermal cells with most of the cell body in the dermis. That they are migrating from the subdermis into the dermis is the only explanation which presents itself; it is

borne out by all the observed facts and contradicted by none. The justification for summing subdermal, dermal, and epidermal melanophores, also included in the tables, follows the same reasoning as that used for the unpigmented cells. Melanophores which appear to be migrating into the dermis are not infrequently seen during the time when unpigmented cells appear to be migrating most actively. Dermal or subdermal melanophores with processes extending into the epidermis are seen, but less frequently.

In *A. tigrinum*, the total number of unpigmented cells slowly increases during larval development and more rapidly as metamorphosis approaches (Fig. 1). The total number of melanophores shows no increase per unit area until after metamorphosis has begun. During advanced metamorphosis there is a rapid increase which corresponds to the time at which the animal rapidly darkens. In *A. maculatum* unpigmented cells increase in number during the larval period, but during metamorphosis the number decreases. The total number of melanophores shows no significant change until metamorphosis begins, but after this the increase is fairly rapid, so that a comparatively high count is obtained in the adult. It is possible that part of the decrease in number of unpigmented cells which appears to occur at this time may be accounted for by their transformation into melanophores. Although there is no direct evidence here to support this suggestion, it has been shown that donor melanophores arise in grafts which previously have been pigment free (DuShane, 1943) and therefore must have differentiated from unpigmented cells. Since mitoses in melanophores are uncommon, the rapid increase in these cells that occurs at the end of metamorphosis

and in the young adult can be explained most satisfactorily as resulting from the transformation of unpigmented cells. One may point out, it is true, that a corresponding decrease in unpigmented cells does not occur in *A. tigrinum*. In *A. tigrinum*, however, the number of unpigmented cells reaches a much higher value, while the increase in number of melanophores is slower and they do not become as numerous as in *A. maculatum*. For these reasons the increase in number of melanophores may not be so obviously reflected in a decrease in number of unpigmented cells as in *A. maculatum*.

The relation between dermal and subdermal cells is indicated in Figures 1 and 2, in which are graphed three-point running averages of some of the data presented in Tables 1 and 2. Mitotic indexes of subdermal and dermal unpigmented cells are also plotted. The increase in dermal thickness that occurs during the developmental period under consideration here are shown in A and B, Figure 4.

T. torosus

The development of the melanophore pattern in this species differs from that found in *Ambystoma*. The first melanophores develop along the back to form a band on either side of the dorsal fin. Melanophores first differentiate in the subdermis and appear in the embryo at about stage 35. Their processes may be found extending along the undersurface of the dermis and at intervals give off fine branches into the epidermis. The development of chromatophores is slightly more advanced in the anterior region. At stage 37, more of the melanophores in the anterior trunk are fairly well pigmented, while more posteriorly they contain fewer pigment granules. This leaves little doubt that melanophores are pigmented *in situ* and do not, as Berweger

(1926) suggested, acquire their pigment only in the anterior part of the body. Epidermal melanophores are found only after those in the dermis have become fairly numerous. By the time the larvae are free-swimming, the subdermal melanophores are fully pigmented and are

very massive, with heavy processes which are packed with large melanin granules.

A peculiar relationship is seen between the subdermal melanophores that form the dorsal bands and the melanophores that are found around the spinal cord.

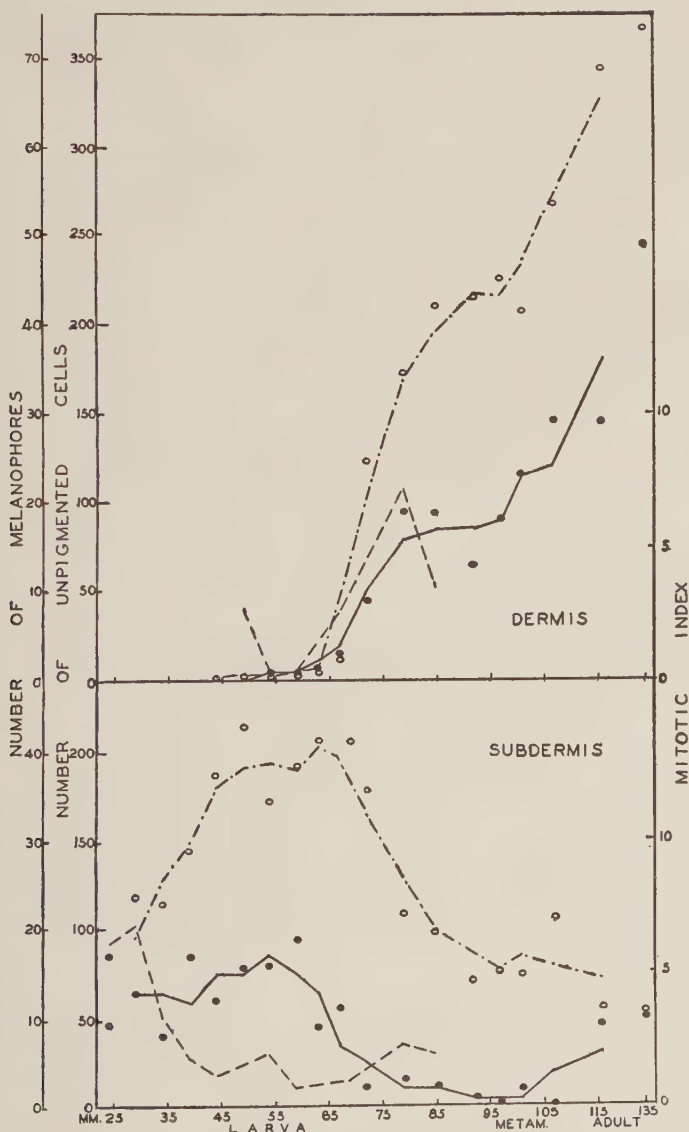


FIG. 1.—*Ambystoma tigrinum*. A three-point running average of unpigmented cells (—.-.—) and melanophores (—) in the subdermis and dermis. Actual values of unpigmented cells (○) and melanophores (●) are plotted (data from Table 1). Mitotic index (-----) of the unpigmented cells of the subdermis and dermis is shown for animals of corresponding stages of development.

The subdermal melanophores occur at the dorsal boundary of the myotomes. These bands are connected to each other and to the melanophores surrounding the spinal cord by other melanophores,

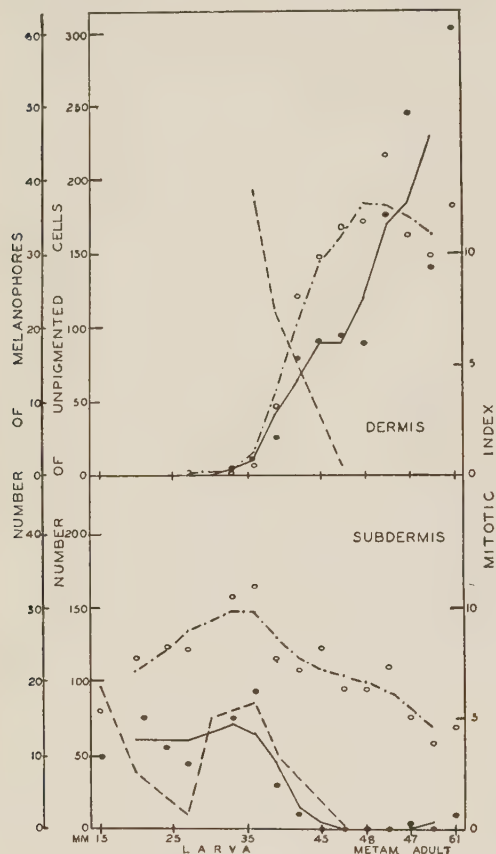


FIG. 2.—*Ambystoma maculatum*. A three-point running average of unpigmented cells (---○---) and melanophores (—●—) in the subdermis and dermis. Actual values of unpigmented cells (○) and melanophores (●) are plotted (data from Table 2). Mitotic index (---●---) of the unpigmented cells of the dermis and subdermis is shown for animals of corresponding stages of development.

which extend over the dorsal margin of the myotomes. In this way a network, sometimes more conspicuous at segmental intervals, is formed over the dorsal surface; but the central part is less apparent in the living animal because of its deeper position in the connective tissue (see A, Pl. II).

This species shows the same general pattern in the formation of the dermis as that found in the species of *Ambystoma* which have been described, but there is some modification in the development of the pigmentary pattern. During the early part of the larval period the heavily pigmented subdermal melanophores occur in the dorsal bands and in a few other well-defined areas on the head and sides of the trunk. In addition, in the subdermal region, a few faintly pigmented melanophores are found which are small and have finely branching processes. These cells contain very little pigment and contribute little to the gross color of the animal. The dermis contains no cells but consists of a layer of connective-tissue fibers about 3–5 μ thick.

As development continues, subdermal cells increase in number until the larva is about 33 mm. in length (see Table 3). At this time the dermis is about 17 μ thick, and cells begin to appear. Soon the subdermal unpigmented cells become less numerous, while dermal cells increase in number, a relation which has come to be expected from the descriptions of the species of *Ambystoma* and which is brought out for *T. torosus* in the graph in Figure 3. Although the melanophores do not change their relative position on the body, they occur at this time primarily in the dermis rather than below it. Again there are many instances of melanophore processes extending into the dermis from below, giving the impression that migration is taking place. Skin-gland anlagen begin to appear at this time also.

There is a slow increase in the number of dermal melanophores during the remainder of the larval period, while unpigmented cells show a more rapid increase. In the advanced larva, shortly before metamorphosis, subdermal unpigmented cells are less numerous, and

melanophores are extremely rare. The dermis is now more than 70μ thick, and the mid-dermal layer contains loosely arranged collagenous fibers, in which numerous unpigmented cells occur. The dorsal bands have become wider, but melanophores are confined, for the most part, to these lines. Skin glands are large and fairly well differentiated, and the dermis extends downward where they

With the onset of metamorphosis, unpigmented cells as well as melanophores occur in the outer portion of the mid-dermal layer where their processes form a network below the epidermis. In many cases dermal melanophore processes extend into the epidermis. The dorsal bands have expanded over a larger area of the back, and melanophores also occur in large numbers over the head and

TABLE 3

RELATION OF THE NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN
LARVAL AND ADULT *Triturus torosus*

LENGTH (MM.)	DESCRIPTION	SUBDERMIS		DERMIS			EPIDERMIS	TOTAL	
		Unpigmented Cells	Melanophores	Unpigmented Cells	Melanophores	Thickness (μ)	Melanophores	Unpigmented Cells	Melanophores
15.....	Larva	57	10	0	0	3	0	57	10
18.....	Larva	74	10	0	0	5	2	74	12
21.....	Larva	103	10	0	0	7	4	103	14
24.....	Larva	88	9	0	0	10	3	88	12
27.....	Larva	111	14	0	0	10	2	111	16
30.....	Larva	117	22	0	0	12	7	117	29
33.....	Larva	126	26	1	0	17	6	127	32
36.....	Larva	134	18	6	1	20	11	140	30
39.....	Larva	121	16	13	2	20	9	134	27
42.....	Larva	101	8	27	9	17	18	128	35
45.....	Larva	78	0	215	11	18	17	293	28
46.....	Larva	43	0	221	13	76	16	264	29
44.....	Metam. I	49	0	280	13	86	29	329	42
49.....	Metam. II	52	0	416	33	111	13	468	46
45.....	Metam. III	46	0	310	33	50	12	356	45
47.....	Adult I	32	0	382	21	51	24	414	45
45.....	Adult II	49	0	309	24	69	44	358	68

occur. Many capillaries penetrate the inner dermal layer and form a network in the loose middle layer. In the advanced larva the epidermis begins to darken and to take on a brownish hue, in contrast to the transparent greenish-yellow which characterizes the young larva. The darkening results from the increase in number of epidermal melanophores, which, although they have been increasing slowly during the larval period, become more numerous at this time. This is accompanied by an increase in the amount of melanin in the epidermal cells.

flanks. The dermis has greatly increased in thickness, but in a more advanced stage of metamorphosis it becomes condensed. These changes in dermal thickness are shown graphically in C, Figure 4. After it has become so condensed, the dermis is no longer wide enough to accommodate the larger skin glands. As a result, the inner dermal layer extends downward around the body of each gland, and the epidermis protrudes outward. In addition, local thickenings of the epidermis occur where the epidermis extends over the glands. It is the aggre-

gate of these two factors which results in the irregular tubercles which are characteristic of adult *T. torosus* epidermis.

Grossly, the young adult appears uniformly brown, and there is no longer any indication of the dorsal bands. Histologically, the subdermal region is unchanged from that found in the advanced larva. The dermis, however, has

become slightly thicker than it was during metamorphosis, approximately $70\ \mu$. The number of dermal unpigmented cells shows what may be a significant decrease in number when compared with the number found during metamorphosis. Dermal melanophores are significantly decreased at this time and are extremely contracted, and the short processes are densely packed with melanin granules. Only rarely can a process be seen extending into the epidermis. These melanophores occur everywhere except on the ventral surface but, because of their extreme contraction, contribute little to the gross coloration of the animal. Some cells are found in the dermis, however, which contain only a few melanin granules. These cells may be developing melanophores, since the nucleus appears normal and the cell as a whole does not show any degenerative changes. The granules are small, more brownish in color, and resemble those of a developing melanophore in the embryo. Epidermal melanophores have again become very abundant and occur as highly branched cells with far-reaching processes. It may be possible to correlate the increase in epidermal melanophores with the decrease in number of dermal melanophores observed at this time. Because of their great expansion, epidermal melanophores may be easily confused with the ordinary epidermal cells, which also contain a large amount of melanin granules. The melanin occurs throughout the cytoplasm of these cells, but it is particularly abundant on the outer side of the nucleus. These characteristics of the adult *T. torosus* skin are shown in B, Plate II.

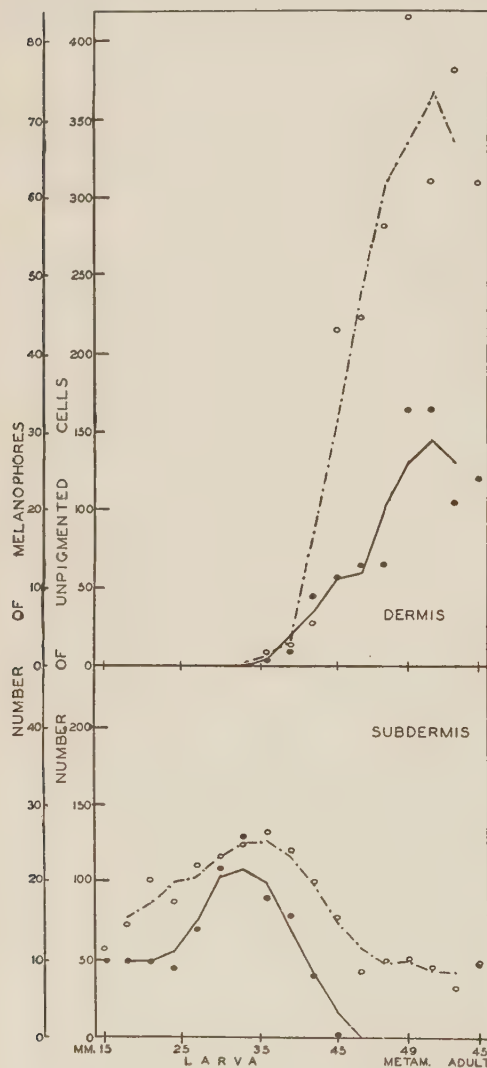


FIG. 3.—*Triturus torosus*. A three-point running average of unpigmented cells (---○---) and melanophores (—●—) in the subdermis and dermis. Actual values of unpigmented cells (O) and melanophores (●) are plotted (data from Table 3).

Table 3 gives the total number of unpigmented cells per unit area—subdermal and dermal—for each animal of the series. There is a slow increase during most of the larval period and a more

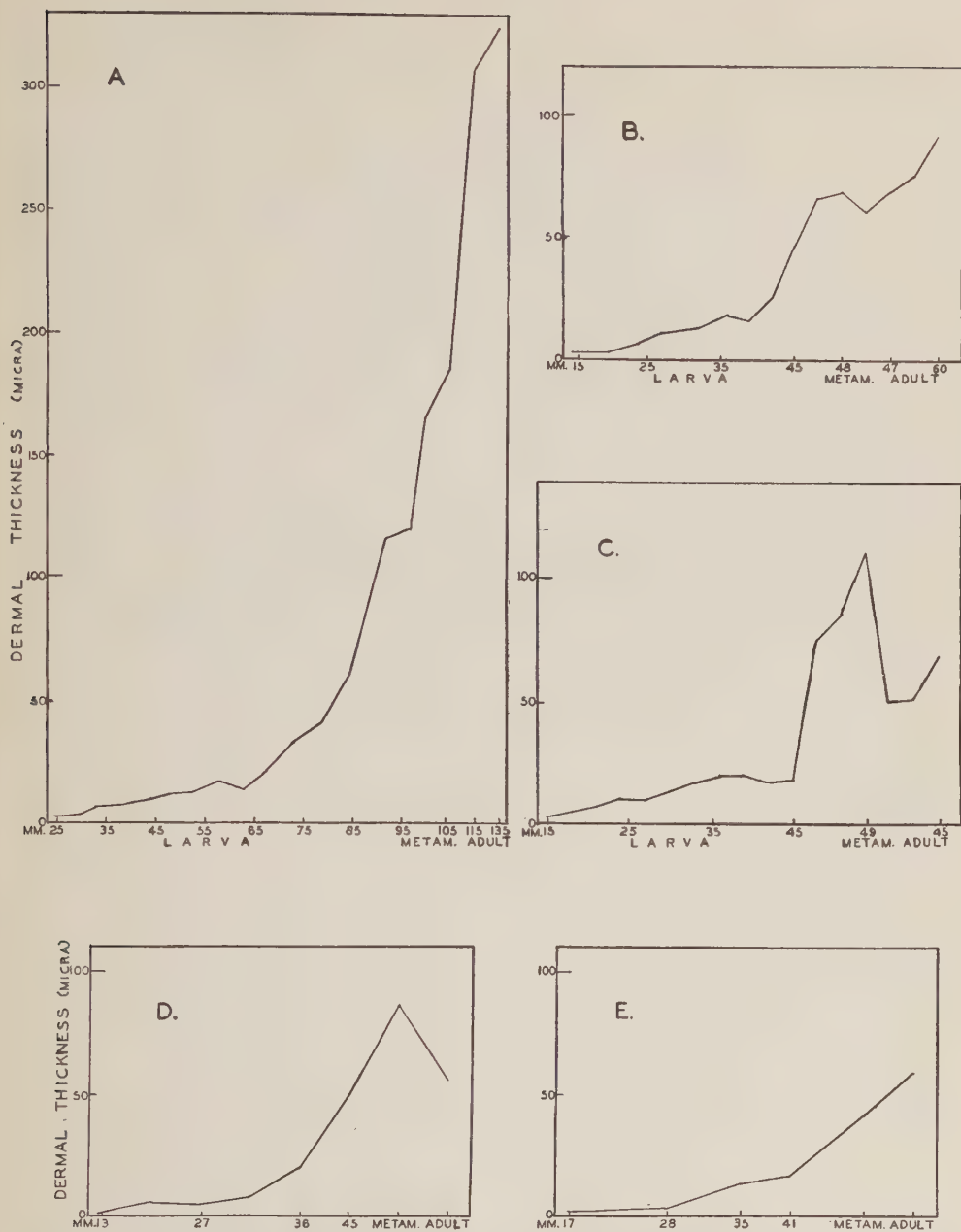


FIG. 4.—The increase in the thickness of the dermis during development in five series. A, *Ambystoma tigrinum*; B, *Ambystoma maculatum*; C, *Triturus torosus*; D, *T. torosus* with grafted neural crest from *A. maculatum*; E, *A. maculatum* with grafted neural crest from *T. torosus*.

rapid one as metamorphosis begins. During the latter part of metamorphosis and in the young adult there is a slight decrease in number of this cell type. Summation of the total number of melanophores per unit area—subdermal, dermal, and epidermal—shows that, while there is a slow increase in number during larval development, the increase is more rapid during metamorphosis and is especially marked when the adult stage is reached. The latter increase is the result of the large number of epidermal melanophores which appears at this time. As in the species of *Ambystoma* which have been described above, it is possible to suggest that here, also, the decrease in number of unpigmented cells may indicate that some have differentiated into melanophores.

EFFECTS OF XENOPLASTIC TRANS-PLANTS OF NEURAL FOLDS

In order to test the effects of the transplantation of melanophores into a strange species, neural folds were transplanted between *A. maculatum* and *T. torosus*. A series of animals was obtained from each combination, from the early larval stage to the adult. As in the normal series, counts were made of a standard area in the region of the graft, to determine the relative proportions of the important cell types. Although it would have been of value to be able to distinguish *A. maculatum* and *T. torosus* unpigmented cells, no consistent difference was found—e.g., in nuclear detail, size, or shape—by which the source of these cells could be determined with accuracy. However, the melanophores of the two species are sufficiently different to permit them to be readily distinguished. Those of *A. maculatum* have more finely branched processes, the melanin granules are not quite so black, and they appear to be smaller. The

processes of *T. torosus* melanophores are shorter and more massive and are packed with intensely black granules.

FROM *A. maculatum* TO *T. torosus*

This series contained ten animals, of which three were allowed to develop to the adult stage. One was fixed while metamorphosing, and the rest represent six stages in larval development.

Larva.—In the youngest animal of this series the graft area occupies the region of the back from between the gills to the mid-trunk. Although the melanophores of the host are limited almost entirely to the yolk border lines and to the dorsal bands, the graft melanophores spread over the entire flanks. Microscopically, the graft area contains melanophores with greatly expanded processes, characteristic of *Ambystoma*. Only a few melanophores are apparent in the host areas at this stage, and they are smaller, with only short processes. The older larvae show little modification in the development of the dermis. Figure 5 shows a larva of 45 mm., in which the graft melanophores form a distinct dark network on the anterior half of the trunk and forelegs. In some animals a few host melanophores are mixed with those of the graft. This mixing is more common in the older developmental stages, and it is doubtful that in the adult there is any pure graft area over the back. As metamorphosis approaches, the graft melanophores increase in number rapidly, and the graft area becomes very dark. The number of dermal melanophores and unpigmented cells increases rapidly, while the number of subdermal cells decreases slowly. The cells of the dermis attain their maximum number during metamorphosis and are slightly decreased when the adult stage is reached. In the metamorphosing animal of this series the graft area is almost black, with a fairly

distinct yellow spot on the back between the gills. Farther posteriorly the host pattern does not show such an advanced stage of metamorphosis. The dorsal bands of melanophores are still clearly visible, and the general color is a greenish brown, in contrast to the uniform brown pattern of the adult *T. torosus*. It has been found in other graft combinations also that the adult *A. maculatum* pattern develops before that of the host. It appears that the threshold of reactivity of grafted cells might be lowered, since, as will be seen later, in the re-

surface. This animal, shown in Figure 6, had the most clearly defined area of donor pigmentation and was therefore used in making the cell counts of the adult (see Table 4, *a*). The graft area invariably showed the normal donor pattern—an intense black background with yellow spots. The spots, however, were not always clearly defined and, although they tended to be smaller, they appeared in the locations which correspond closely to those in the normal donor, as nearly as can be determined without detailed study. In the host area the pattern was

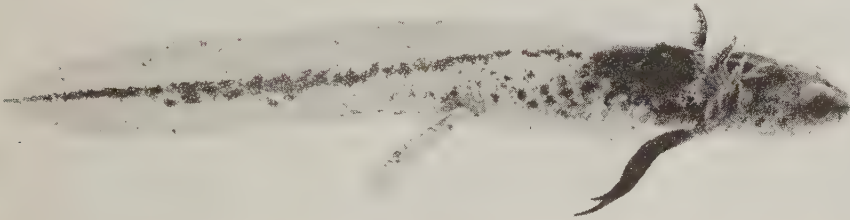


FIG. 5.—Advanced *T. torosus* larva (45 mm.) with neural crests transplanted from *A. maculatum*. The graft melanophores form a black network over the anterior trunk region and the forelegs, while the host melanophores in the posterior trunk and tail form the dorsal bands characteristic of *T. torosus*.

ciprocal graft combination *T. torosus* melanophores assume the adult pattern before the transformation is complete in the *A. maculatum* host. Further work is needed to determine the factors responsible for this phenomenon.

Postmetamorphosis.—Three animals of this graft combination were raised to young adults. In all cases the graft area extended over the posterior part of the head and the anterior half of the trunk, in two cases the forelegs showed the characteristic black of the donor, and in one case the graft melanophores extended to the mid-ventral line, so that a black strip was formed across the ventral surface between the forelegs. This is in sharp contrast to the normal host pattern, in which melanophores never occur on the ventral

that of the normal adult *T. torosus*. The tail and posterior trunk in these cases showed no graft melanophores but only the uniform brown, characteristic of the host. Microscopic study of the graft area shows that the dermis contains a large number of melanophores, which are highly branched and occur in the loose connective tissue of the middle layer. Rarely, a contracted melanophore can be seen, characteristic of the adult host; but much less epidermal pigment is present than is found in the normal *T. torosus*. In the mid-dorsal region a small area of graft epidermal cells (from *A. maculatum*) contains less melanin than the surrounding host cells (see *D*, Pl. II). The skin glands which have formed from the graft epidermis are characteristic of

the donor and show consistent differences from those of the host. The dermis in the area of host pigmentation contains only a few melanophores which are greatly contracted, although a few fine processes extend into the epidermis. More melanin is present in the epidermal cells than was found in the area of graft melanophores.

Table 4, *a*, gives the actual values obtained from cell counts in the animals in this series. That the relation between important cell types is unchanged from that found in the normal series can be

young adult stage, one was fixed while undergoing metamorphosis, and the remaining four were fixed at representative stages in larval development. In only one or two instances was it possible to obtain a graft area of *T. torosus* cells which was not invaded by melanophores of the host. Apparently, *A. maculatum* cells have a greater capacity to migrate, and they invade areas of *T. torosus* cells very readily.

Larva.—The youngest animal of the series shows a clear graft area over the entire trunk. Melanophores on the dor-



FIG. 6.—Adult *T. torosus* with neural crests transplanted from *A. maculatum*. Graft melanophores are evenly distributed over the anterior trunk region and forelegs. Note the small spot, characteristic of *A. maculatum*, on the left shoulder.

seen in Figure 7. From this graph it is apparent that the strange host environment has essentially the same influence upon the graft cells as has their normal environment. Dermal thickness, included in Table 4, is graphed in *D*, Figure 4. It can be readily seen that dermal development, if increase in thickness is used as a criterion, closely follows that found in the normal *T. torosus*. It is apparent, therefore, that the presence of foreign (graft) melanophores, as well as unpigmented cells within the dermis, does not alter its normal pattern of development.

FROM *T. torosus* TO *A. maculatum*

Of the six animals with this graft combination, one was carried to the

sal surface form fairly distinct dorsal bands, and almost no epidermal melanophores are present in this area. Histologically, graft melanophores are densely pigmented and somewhat contracted, in contrast to the larger, more expanded host melanophores. The next animal of the series has an indistinct graft area. The trunk contains a few dermal melanophores, which are darker and more contracted than the surrounding host melanophores. There is no essential change in histological detail when compared to the normal, and there are probably very few graft cells in the area. In the more advanced larvae of the series the graft area is somewhat clearer, although some chromatophores are present. Grossly, the dorsal bands are

somewhat indistinct, and the area around them is lighter than the corresponding area in the host (fewer and more contracted melanophores) but darker than is found in the donor of the corresponding stage of development. The dermis develops normally, but the

is reached in the host. A satisfactory explanation of this phenomenon must await further investigation. Nevertheless, this gray donor pattern is not quite characteristic of *T. torosus*. Microscopic study shows that the dermis contains melanophores which are almost com-

TABLE 4

GRAFT SERIES: RELATION OF NUMBER OF MELANOPHORES AND UNPIGMENTED CELLS PER UNIT AREA TO THE DEVELOPMENT OF THE LAYERS OF THE SKIN IN LARVAL AND ADULT ANIMALS

LENGTH (MM.)	DESCRIPTION	SUBDERMIS		DERMIS			EPIDERMIS	TOTAL	
		Unpigmented Cells	Melanophores	Unpigmented Cells	Melanophores	Thickness (μ)	Melanophores	Unpigmented Cells	Melanophores
a) <i>T. torosus</i> with <i>A. maculatum</i> neural crest									
13.....	Larva	77	13	0	0	2	1	77	14
21.....	Larva	98	13	0	0	6	4	98	17
27.....	Larva	135	12	0	0	5	2	135	14
31.....	Larva	127	12	0	0	8	5	127	17
36.....	Larva	83	14	21	8	20	3	104	25
45.....	Larva	73	6	141	38	50	20	214	64
51.....	Metam.	92	12	458	86	86	10	550	108
49.....	Adult	18	4	280	77	56	19	298	100
b) <i>A. maculatum</i> with <i>T. torosus</i> neural crest									
17.....	Larva	80	8	0	0	2	1	80	9
28.....	Larva	140	9	0	0	3	5	140	14
33.....	Larva	95	7	33	3	13	2	128	12
41.....	Larva.	97	4	44	19	16	6	141	29
49.....	Metam.	73	4	154	50	41	1	227	55
58.....	Adult	83	4	252	44	59	4	335	52

larval pigmentary pattern of the donor does not develop to the extent seen in the reciprocal graft combination.

In the animal which was fixed during metamorphosis, the graft area was clearly defined and extended over the entire trunk. At fixation, this region was a light gray, except for two or three areas over the back, which were darker and evidently contained host chromatophores. Here, again, the graft attains the adult pattern before this stage of development

pletely contracted. Almost no pigment is present in the epidermal cells, and epidermal melanophores are extremely rare. It is the absence of epidermal pigment, as well as the presence of only contracted melanophores in the dermis, which is responsible for the gray appearance of the graft area.

Postmetamorphosis.—The only adult in this series shows a small but fairly distinct graft area on the back between the forelegs, as well as on the forelegs

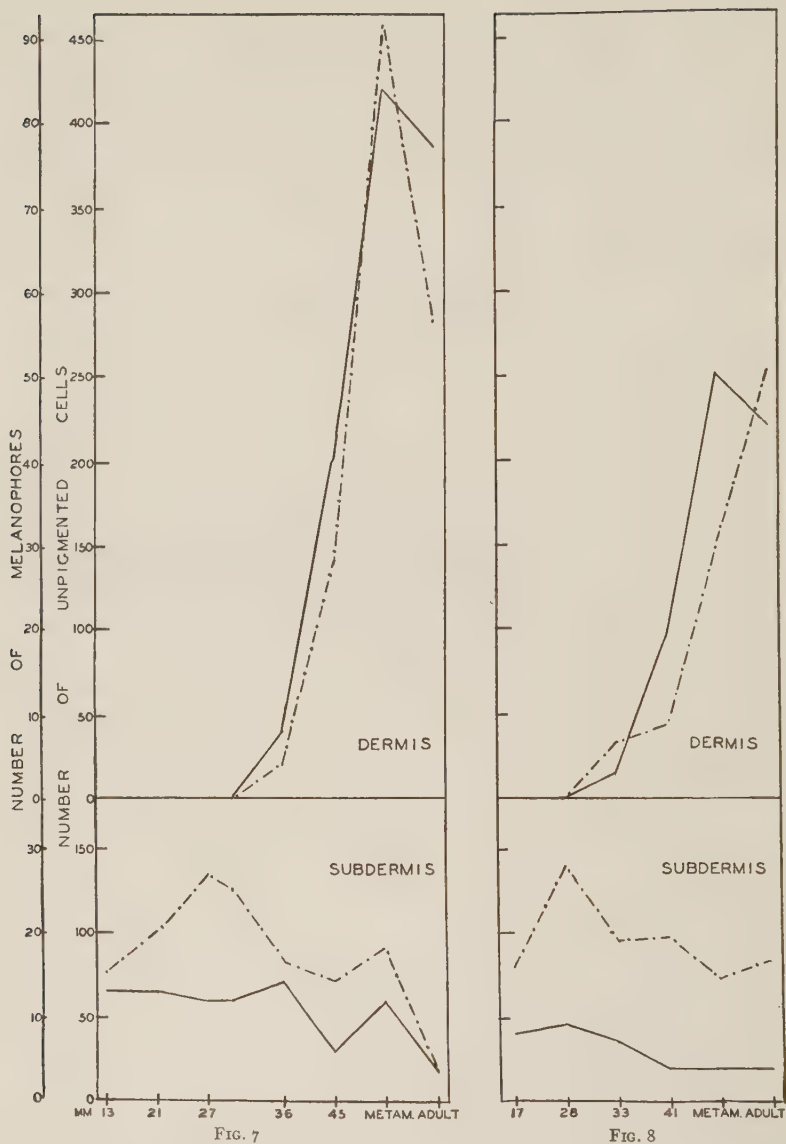


FIG. 7.—*Triturus torosus* with neural crests grafted from *A. maculatum*. Curves show the number of unpigmented cells (---) and melanophores (—) in the subdermis and dermis for each animal in the series.

FIG. 8.—*Ambystoma maculatum* with neural crests grafted from *T. torosus*. Curves show the number of unpigmented cells (---) and melanophores (—) in the subdermis and dermis for each animal in the series.

themselves. This area on the body appears light gray, and the legs are even lighter. The surrounding host skin is quite black, and distinct yellow spots are evident. Grossly, the grafted melanophores in the dermis appear as dots, while histologically they are seen to be extremely contracted. Expanded melanophores also occur in this area and are probably of host origin. As in the preceding animal, the epidermis in the area of the graft melanophores is almost completely devoid of melanophores, as well as melanin, in the epidermal cells. However, a few donor epidermal cells which are present in the mid-dorsal region contain large amounts of melanin granules (see *C*, Pl. II). The forelegs are even more free of host melanophores and epidermal pigment and therefore appear even lighter in color. Table 4, *b*, lists the values obtained in cell counts in the animals of this series. Although the number of animals is small, Figure 8 shows that the relation between dermal and subdermal unpigmented cells and melanophores is essentially the same in this graft combination as has been found in the normal host, *A. maculatum*. The graft cells appear to have no influence upon the development of the dermis, at least not when thickness is used as a criterion.

EPIDERMAL PIGMENTATION

The origin of epidermal melanophores, as well as the source of pigment in the epidermal cells, has been the subject of much controversy. For this reason special attention has been given to this point in the material available in the present study. Many instances have been found of the migration of melanophores into the epidermis from the deeper layers of the skin, and there is a good correlation between the presence of expanded melanophores and the occurrence of melanin in epidermal cells.

As has been mentioned briefly above, the epidermis of the adult *T. torosus* contains large amounts of melanin, both in epidermal cells and in melanophores. This condition is attained by a developmental process which extends throughout the larval period (see *A*, Fig. 9). In the embryo, melanophores first occur only in the subdermal region, at a time when only egg pigment is present in the epidermal cells. Although this pigment may be confused with the pigment occurring in melanophores, the two may be distinguished by the fact that the melanin granules found in chromatophores do not occur in such a finely divided state as does egg pigment. In the larva and adult the melanin occurring in epidermal cells is more likely to be accumulated in a particular part of the cell than to be evenly distributed throughout the cytoplasm. The major part of this melanin is usually aggregated to form a cap over the outer side of the nucleus, while the remaining granules are distributed more evenly and sparsely in the rest of the cytoplasm. Soon after subdermal melanophores begin to appear in the *T. torosus* embryo, their processes are seen extending into the epidermis. This is followed by an increase in epidermal pigment, especially in the regions around melanophore processes. However, only a few epidermal melanophores and little epidermal melanin is present during the early larval period, and it is not until the larvae reach a length of 30–35 mm. that epidermal melanophores become much more numerous. There is little epidermal pigment in the vicinity of contracted melanophores; but, when the melanophores are expanded, the epidermal cells contain more pigment. In the older larvae there is a further increase in epidermal melanophores and in the melanin found in epidermal cells. These

melanophores are extremely expanded, and their fine, highly branched processes extend for long distances between the epidermal cells. At their ends many of the processes penetrate the epidermal cells and pass over the outer side of the nucleus to form a cap. In many instances it is possible to see a fine line of granules at the end of the melanophore process.

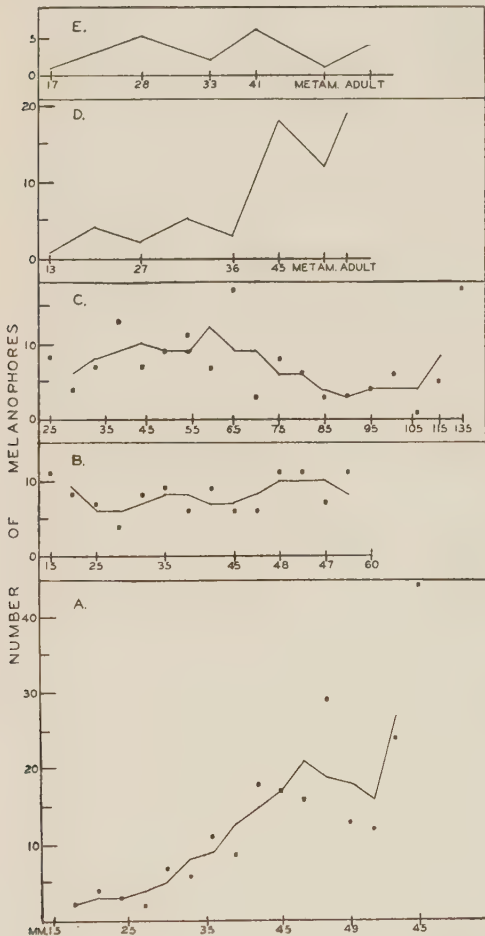


FIG. 9.—Curves show the variation in number of epidermal melanophores during development. A, *T. torosus*; B, *A. maculatum*; C, *A. tigrinum*; D, *T. torosus* with grafted *A. maculatum* neural crests; E, *A. maculatum* with *T. torosus* neural crests. In A, B, and C, the graph represents a three-point running average of actual values, while the dots represent observed values of each animal in the series (data from Tables 1, 2, and 3). In D and E the graph shows actual values (data from Table 4).

This condition is seen perhaps best in the advanced *T. torosus* larva (see E, Pl. II) at the time when epidermal pigmentation is increasing most rapidly. At the beginning of metamorphosis the epidermis is heavily pigmented, and the relation of numerous epidermal melanophores, and much epidermal pigment which is present at this time persists throughout adult life. As for the source of epidermal melanophores, one may find evidence of migration of melanophores into the epidermis from deeper positions during the entire larval period, but it is most commonly seen at the time when the number of epidermal melanophores is increasing most rapidly.

In the other species studied here, there is no regular development of epidermal pigmentation as in *T. torosus*. In *Ambystoma* some epidermal melanophores are always present, but there is considerable variation in number in the larva as well as in the adult (see B and C, Fig. 9). When the epidermal melanophores are contracted, there is usually a complete absence of epidermal pigment, while, when they are highly expanded and many processes enter the epidermal cells, there is a large quantity of epidermal pigment. In all cases epidermal pigment can be found only in the part of the epidermis in which melanophores are present. It has also been noted that epidermal melanin is more abundant where many melanophore processes enter the epidermal cells. Tangential sections of *A. tigrinum* and *A. maculatum* epidermis show finely branched melanophores with processes penetrating many of the surrounding epidermal cells. These processes appear to break up within the epidermal cells and to form a cap over the outer side of the nucleus. (In sections stained with thionine, it is seen that lipophores as well as melanophores send finely branched processes for some dis-

tance between the cells of the epidermis and commonly enter these cells, as do the processes of the melanophores.)

As further evidence of the relation between epidermal pigment and melanophores, a study was made of other species in which a special attempt was made to compare dorsal and ventral epidermis. In *T. pyrrhogaster* the ventral skin usually contains no epidermal melanophores, and there is no pigment in the epidermal cells. However, one small epidermal melanophore was found, and the epidermal cells for a short distance around it contained a small amount of melanin granules. *Triturus granulosa* also shows no epidermal melanophores on the ventral surface and no melanin granules in the epidermal cells. On the flanks an occasional melanophore may be found, as well as some epidermal pigment, while farther dorsally more epidermal melanophores are present and there is a large amount of epidermal melanin. Again, in *T. rivularis*, there is a high degree of correlation between the presence of epidermal pigment cells and epidermal pigment.

In the animals with grafted neural crests there is additional evidence of the relation between epidermal cell pigment and melanophores. The area of graft melanophores in adult *T. torosus* with neural crests from *A. maculatum* contains, in most cases, a few host melanophores, but much less than is normally found. It is relatively easy to differentiate host and donor dermal melanophores, since those from *T. torosus* are always extremely contracted in the adult, while those from *A. maculatum* are expanded. However, it was possible to obtain only graft chromatophores in the forelegs of one adult *T. torosus* host, and in this case there was no evidence of the migration of grafted *A. maculatum* dermal melanophores into the epidermis, and neither

epidermal melanophores nor epidermal pigment was found there. Where a small area of graft epidermis is present (in the mid-dorsal cervical region), these cells contain the normal amount of melanin, and melanophores are present. The normal pigmentation in the graft epidermis indicates that this phenomenon is not the result of some effect of the strange environment of the host upon the graft melanophores. In *D*, Plate II, the sharp line of demarcation between the epidermis of graft and of host origin is shown. The donor epidermal cells contain a small amount of epidermal pigment, characteristic of normal *A. maculatum*, while the melanin in the host epidermis is strikingly reduced as compared to the normal host, *T. torosus*. Counts of epidermal melanophores in this series, graphed in *D*, Figure 9, show that there is an increase in number during development similar to that found in the normal *T. torosus* but that in the graft animals the increase is not so great. The small number of host melanophores, which are present in the region dominated by graft melanophores, is probably the source of the slight increase in epidermal pigment which is observed.

The reciprocal graft combination illustrates equally well the inability of graft melanophores to pigment the epidermis of the host. Although the melanophores of *T. torosus* are normally able to produce the heavily pigmented epidermis, only a few epidermal melanophores and a small amount of epidermal pigment are present where these melanophores are covered by host (*A. maculatum*) epidermis. In *C*, Plate II, the same relation as that noted above is shown. The small area of graft epidermis is heavily pigmented, while the adjoining host epidermis is essentially pigment-free. The counts of epidermal melanophores recorded for this series are

graphed in *E*, Figure 9, and show that the course of development follows that expected for the normal host, *A. maculatum*. The number of melanophores is not so great as that found in the normal animals of corresponding stages of development. It will be recalled that a small number of host melanophores are typically present in an area of graft melanophores and that these host cells may well be the source of the reduced number of epidermal melanophores which are present.

Further explanation of these findings will be reserved for the discussion.

DISCUSSION

A study of the development, of cutaneous pigmentation and of the associated development of the dermis indicates that metamorphosis is only one step in the continuous process which is begun with the formation of the individual. All processes of growth and differentiation are continuous and can be divided only arbitrarily into stages. Although certain external changes which accompany the transfer from a water to a moist land habitat are fairly well demarcated, changes in the pigmentary pattern are more gradual, and dermal development is completed before metamorphosis is begun.

Recent investigations have reopened the controversy concerning the elements contributing to the development of the dermis. Detwiler (1937) found that the corium of the skin is completely developed on the side of the body from which the somites have been removed. He also followed migrating neural-crest cells, which had been vitally stained, to a position between the somites and the developing skin. From these results he was led to the conclusion that at least a part of the dermis is formed from the neural crest. Holtfreter (1935) and Raven

(1936) also reached this conclusion with similar types of experiments. From the present study further evidence can be added in support of this hypothesis. The cells which occur immediately beneath the dermis in young larvae may be those which Detwiler, in his vitally stained preparations, observed to migrate from the neural crest. This investigation has shown that the cells which lie just below the dermis in the young larva appear to become the cells of the dermis later in development. At no time is it possible to differentiate by morphological criteria the unpigmented cells of the subdermis from those of the dermis. Although morphological similarity is no indication of identical origin or of a similar fate, it may be noted that there is no difference in the cytology of the nuclei of melanophores and unpigmented cells.

Unpigmented cells occur in close association with the pigment cells which DuShane (1935) has proved to have a neural-crest origin. DuShane (1935, 1943) has shown experimentally that potential melanophores migrate in the undifferentiated state and become pigmented *in situ*. The evidence presented here indicates that at least some of the unpigmented cells of the subdermis and later of the dermis are potential pigment cells. Some differentiate before they migrate into the dermis, but the majority enter in the undifferentiated state and only later elaborate melanin. The graphs show that the ratio of pigmented to unpigmented cells is smaller in the advanced larva than it is after metamorphosis is completed. During metamorphosis the number of melanophores rapidly increases, while the unpigmented cells show a general decrease. In the adult many unpigmented cells are present among the melanophores of the dermis, and at least some of these must

represent potential melanophores. Although mitoses have been observed in differentiated pigment cells, this is not common and would contribute little to the rapid increase which takes place during metamorphosis.

Specific factors inherent in the pigment cells are of major importance in determining the pattern of distribution that is specific for the species. As has been pointed out by Twitty (1936, 1944) and Twitty and Bodenstein (1939, 1944), interspecific neural-crest grafts have shown that the melanophores of *T. torosus*, which normally form two dorsal bands, can form this characteristic pattern regardless of the environment. More recently (1945), Twitty has attempted to determine the factors present in *T. torosus* melanophores which are responsible for their aggregation at the dorsal margin of the myotomes to form the dorsal bands. From observations on explants and on transplants between *T. torosus* and *T. rivularis*, he concluded that the characteristic behavior of *T. torosus* melanophores is the result of their ability to attain a degree of differentiation higher than that observed in other species. He considers that this high degree of differentiation is necessary for the reaggregation of these cells into the dorsal bands. In the forms in which melanophores are more uniformly distributed, this high degree of differentiation is not reached.

Reciprocal graft combinations between *A. maculatum* and *T. torosus* show that, when the host and donor are the same age, melanophores of the former have a greater capacity for migration. *Ambystoma maculatum* grafts on *T. torosus* tend to be larger and to maintain themselves more clearly than do the reciprocal ones. *Triturus torosus* grafts on *A. maculatum* tend to be invaded by host cells, so that in the adult it is extremely

difficult to obtain an area of pure donor melanophores. It has been observed, however, that, if older *T. torosus* grafts are transplanted to younger *A. maculatum* hosts, a larger graft area is obtained which is relatively more free of host cells. This relationship has been noted by Twitty and Bodenstein (1944), who observed that relative age differences between host and donor influence the extent of migration of neural-crest cells. In addition to these intrinsic factors, the surrounding tissues exert a definite influence upon the potentials of chromatophores. A number of workers have shown the influence of epidermis, as well as of somites and neural tube, upon the melanin-forming capacity of these cells (DuShane, 1935, 1939; Twitty, 1936; Twitty and Bodenstein, 1939; and DeLanney, 1941).

The developmental changes in pigmentation in the skin and subcutaneous regions, which have been described in this investigation, indicate that in urodeles a simple relation exists between subdermal, dermal, and epidermal melanophores. Berweger (1926) has described the development of pigmentation in *Salamandra maculosa*, and in this form she observed migration of melanophores from the cutis into the epidermis in the embryo, larva, and adult. It appears most probable, then, that all these cells arise from one cell type and that the only distinction which could be made would be one based on their position at a given time.

More recently, however, Elias (1939, 1941, 1942) has proposed an elaborate classification for melanophores, based on his discovery of certain colorless pigment cells in the dermis of the toad, *Bombinator pachypus*. The system is set forth for the amphibians in general, and he considers it to hold for urodeles as well as anurans. He recognizes four types, dis-

tinguished according to their location: (1) epidermal melanophores, found in the epidermis and occurring in all amphibians with the exception of the adults of *Bombinator* and *Hyla*; (2) adepidermal melanophores, found immediately below the marginal layer of the epidermis and occurring in urodeles and certain anurans; (3) intercutaneous melanophores, found deeper in the dermis and occurring in the adults of other anurans; and (4) subdermal melanophores, occurring in the larvae of all anurans. This scheme indicates that urodeles, throughout their life-history, possess melanophores only in the epidermis and in the dermis immediately below the epidermis and that subdermal melanophores never occur in this group. In the urodeles studied in this investigation subdermal melanophores were invariably found in the younger larvae. It is only in the older urodele larvae that melanophores are found in the dermis, while subdermal melanophores may continue to be present in reduced numbers throughout the life of the animal. Elias makes no distinction between the melanophores of the young and old urodele larva but considers that they are of only one type and occur in one position, adepidermally. This is in contrast to the condition which he describes in anurans—i.e., the degeneration of subcutaneous melanophores at metamorphosis and the new formation of dermal ones. Because of this difference, Elias homologizes the dermal melanophores of urodeles not to the dermal melanophores of anurans but to the adepidermal ones, which he found to persist throughout larval life in the discoglossids. In view of his misinterpretation of the condition existing in urodeles, one may be inclined to reject his distinction between the melanophores found in the adults of urodeles and anurans and to consider all melanophores occurring in

the dermis to be of one cell type. This leaves the three types of melanophores which have been recognized here in relation to their position in the skin: subdermal, dermal, and epidermal.

A histological study of the development of the pigmentary pattern reveals that many changes take place before the onset of metamorphosis. The greatest structural changes occur before the animal leaves the water, although, grossly, little change is noted in the living animal during this time. In the species of *Ambystoma* studied, the striking color change at metamorphosis is brought about by an increase in number of melanophores and their rearrangement within the dermis to form a more complete layer instead of a loose network. Further rearrangement of the melanophores leads to the formation of spots. They are formed by the aggregation of lipophores in the epidermis and the disappearance or emigration of epidermal melanophores from these areas. Contrary to the report by Schmidt (1919) on *S. maculosa*, in *Ambystoma* some dermal melanophores do remain under the epidermis in a spot area, but they are less numerous and are more loosely arranged in the mid-dermal layer. There is no hint in the literature as to the local differences which are responsible for spotting. The melanophores themselves must contain some of the factors, since *A. maculatum* chromatophores form spots in *T. torosus* hosts, which normally are uniformly pigmented. In *T. torosus*, the pigmentary changes which occur in the dermis during metamorphosis are masked to a large extent by the heavy pigmentation of the epidermis, which develops at this time. Although the dermal pigmentary changes are of importance in forming the adult pattern, it is the epidermal pigment which plays the major role in the pigmentation of the adult in this species.

In evaluating the results of the graft series, it is of interest to compare the normal pigmentation and dermal development in the two species involved. The course of development has been shown to follow the same general trend, and comparison of the total number of melanophores (subdermal, dermal, and epidermal) and unpigmented cells (subdermal and dermal) per unit area in *A. maculatum* and *T. torosus* (Tables 2 and 3) shows that the number present at comparable stages of development is similar. However, during metamorphosis the number of unpigmented cells in *T. torosus* becomes greater than that found in *A. maculatum*. From these data it is impossible to state what difference is a significant one; hence it can be said only that the value in the adult *T. torosus* is about one-third larger than that found in *A. maculatum*.

Counts of the total number of melanophores (subdermal, dermal, and epidermal) and unpigmented cells (subdermal and dermal) per unit area in the two graft series (Table 4) show that the course of development is very similar. No conclusion can be drawn with regard to the source of the elements contributing to the dermis, since the numbers found may be considered normal for either species and the melanophores maintain themselves as well in the graft as in the normal environment. If it is assumed, as has been suggested above, that the unpigmented cells, at least a part of which are potential pigment cells and which participate in the formation of the dermis, are derived from the neural crest, then it is seen that these cells are likewise not adversely affected by the strange environment of the host. However, in the case of the metamorphosing and the adult *T. torosus* with *A. maculatum* grafts, the number of melanophores is far in excess of that found in the nor-

mal animals of this series. It is possible that this is partially due to the inability of donor melanophores to penetrate the epidermis of the host where they would normally give off their pigment and so become depleted. The suggestion is made that the donor melanophores accumulate in the dermis, and this results in the increased number. Still another factor may be a difference in threshold of reactivity of the two types of melanophores. *Ambystoma maculatum* melanophores may be responding to a higher level of melanogenic substance in the *T. torosus* host. The latter is the least probable, however, since it is known that the tissues of *T. torosus* are relatively poor in the ability to promote melanin formation (DeLanney, 1941). Further work is needed to bring out these relationships.

Attempts have been made in these observations to determine the origin and formation of epidermal pigment. Although epidermal melanophores do not develop in the absence of the neural crest, DuShane (1935) states that decisive evidence of their source is lacking. No morphological difference was present in the epidermal melanophores of the different species that he used, so xenoplastic grafts throw no light on their origin. The fact that dermal melanophores are the first to develop and that they are so commonly seen migrating into the epidermis gives support to the hypothesis that epidermal as well as dermal melanophores are derived from the neural crest. Migration of dermal melanophores into the epidermis occurs throughout the larval period and is particularly prominent in the advanced larva and during metamorphosis in *T. torosus*, at the time when epidermal melanophores increase most rapidly. The evidence seems to indicate that the morphology of melanophores may be modified by the epidermal environment.

After metamorphosis, epidermal melanin granules in *T. torosus* become finer, while epidermal melanophores become more finely branched and contain finer granules. It appears that these changes take place in the melanin which is already present in the epidermis, since there is no indication that this peculiar melanin is brought to the epidermis from elsewhere at this time. The epidermal environment may also modify the morphology of epidermal melanophores, and, as a result, it may not be possible to judge the source of these cells by their morphology.

DuShane (1938) has shown that in animals with extirpated neural crests, dermal melanophores migrated into the unpopulated trunk region before epidermal melanophores appeared. This is the time-relation expected if epidermal melanophores have their origin in the neural crest, as do the dermal ones.

The significance of the melanin that occurs in the epidermal cells has been interpreted in a variety of ways. In some cases it has been confused with egg pigment and so has been considered a product of metabolism. Berweger (1926), in her study on *S. maculosa*, has never observed the penetration of melanophore processes into epidermal cells and has frequently found epidermal pigment in areas in which no chromatophores occurred. She concludes, therefore, that epidermal cells have the capacity to form melanin independently of the presence of melanophores. Few investigators have considered this melanin to be derived from the melanophores, which extend their processes for long distances between the epidermal cells (e.g., Ehrmann, 1885) and, in the cases observed here, without any doubt do enter these cells. The present observations have shown that there is a close correlation between the presence of epidermal melanophores and melanin in epidermal cells.

In the species studied here, epidermal pigment occurred only in regions where epidermal melanophores could be found. If the melanophores are greatly contracted, it is unusual to find much epidermal pigment, while, when they are expanded and their processes penetrate the epidermal cells, a great deal of melanin may be present in these cells.

The neural-crest graft series supply the best evidence of the relation between melanophores and pigment in the epidermis. Observations on these series make it evident that the donor melanophores do not migrate into the epidermis of the host and that in their absence no melanin is present in the epidermis. As a result, neither epidermal melanophores nor epidermal pigment occur in the epidermis covering an area containing only graft chromatophores. It appears that these pigment differences result entirely from factors present in the epidermis, since an area of graft epidermis adjacent to the unpigmented host epidermis contains the normal amount of melanin. These results strongly indicate a common origin of dermal and epidermal melanophores and make highly probable a relation between epidermal melanophores and the pigment in epidermal cells. It must be understood, however, that these statements are made only for the species which have been investigated; generalizations must await descriptions of the conditions which hold in a wider variety of amphibians.

Studies of other classes of vertebrates have revealed that the relation between melanophores and epidermal pigment which has been suggested here is not peculiar to amphibians. In birds it has long been known that the melanophore releases its melanin granules to the barb and barbule cells of the developing feather (e.g., Aeby, 1885; Strong, 1902; Willier and Rawles, 1938, 1940; and,

most recently, Watterson, 1942). It is by this release of pigment from the chromatophore that the feather becomes pigmented. The pigment cell then degenerates so that the entire pigmentary function in the adult is taken over by the feathers. Here, then, there is a greater specialization in the function of the epidermal melanophores than that found in amphibians. It has been shown, however, that there is a reservoir of potential pigment cells, which differentiate in the development of regenerating feathers (Foulks, 1943).

In mammals recent work indicates that melanophores may supply pigment to the epidermal cells of the developing hair follicle. Rawles (1940) took pieces of ectoderm with adhering mesoderm from over the neural crest, as well as from the limb buds, of $8\frac{1}{2}$ –12-day mouse embryos. When transplanted to the coelom of White Leghorn host embryos, all grafts developed skin and hair follicles; but grafts from the hind-limb bud contained no pigment cells in the follicles and the hairs were entirely without pigment. Aeby, as early as 1885, stated his belief that epidermal pigment always came from chromatophores and that the epidermis was unable to form pigment in any class of vertebrate.

In birds the origin of epidermal pigment from melanophores has been clearly demonstrated. The same relationship appears to hold in mammals, although as yet it has not been so conclusively demonstrated. In amphibians indisputable proof on this point is still lacking, but in the light of evidence in the other classes of vertebrates it would not be surprising to find the same relation.

SUMMARY AND CONCLUSION

The development of pigmentation and of the dermis is described in three

species of urodeles—*A. tigrinum*, *A. maculatum*, and *T. torosus*. The changes in the dermis and in the pigmentation in *A. maculatum* and *T. torosus* with reciprocal neural-crest grafts are given special consideration, as well as the problem of the source of epidermal pigment.

THE DEVELOPMENT OF THE DERMIS

1. In the young larva the dermis consists of a thin layer of collagenous fibers which contains no cells. As development proceeds, the dermis increases in thickness and soon contains unpigmented cells and melanophores. The cells apparently originate by migration from the subdermis. Subdermal cells are most numerous just before dermal cells appear. As dermal cells increase in number, the cells of the subdermis become less numerous.

2. Skin glands begin to form in the advanced larva and are large and well differentiated when metamorphosis is completed. The striking increase in dermal thickness which occurs in the advanced larva is associated with the rapid increase in the size of these glands.

3. The unpigmented cells of the subdermis and dermis resemble undifferentiated mesenchyme cells. From indirect evidence it seems certain that at least some of these cells are melanoblasts, and it has been shown that they contribute to the formation of the dermis. Since melanoblasts are known to have a neural-crest origin, the origin of the other cells of the dermis from this source also is highly probable. The neural-crest graft series give no information concerning the origin of the dermis, since the mode of development is similar in the two species which were employed (*T. torosus* and *A. maculatum*).

THE DEVELOPMENT OF PIGMENTATION IN THE DERMIS

1. In the young larva, melanophores are found below the dermis and in the epidermis. Later in larval development they occur in the dermis also, apparently having migrated from the subdermis. During metamorphosis and in the adult, melanophores are rarely found in the subdermis but occur principally in the dermis and the epidermis.

2. In the two species of *Ambystoma*, changes in the external appearance of the pigmentary pattern which occur at metamorphosis result from an increase in number of dermal melanophores and their aggregation below the epidermis to form a fairly complete layer. This results in the black background which is characteristic of these species. Spots result from the aggregation of lipophores in the epidermis and the slight decrease in number of melanophores in the underlying dermis.

3. In *T. torosus*, metamorphosis is characterized by the development of a uniform brown pattern. This is brought about by a large increase in number of epidermal melanophores and melanin

during metamorphosis and a contraction of dermal melanophores.

4. Grafted melanophores are capable of maintaining themselves in the environment of a foreign species and of reproducing the donor pattern.

THE DEVELOPMENT OF EPIDERMAL PIGMENTATION

1. Epidermal melanophores have highly branched processes, which extend for long distances between the epidermal cells. At their ends the processes can enter the epidermal cells, where they pass over the outer side of the nucleus and appear to break up. In animals with inter-specific neural-crest grafts, donor melanophores appear to be unable to migrate into the epidermis of the host. No melanophores or epidermal pigment are present in the epidermis overlying these areas of donor melanophores.

2. The intimate relation between melanophores and epidermal cells suggests that melanophores are the source of the melanin that occurs in the epidermis. This relation has been shown to be true in birds, and the evidence in mammals, although not so conclusive, is strongly suggestive of the same conclusion.

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PLATE I

The development of the skin in *A. tigrinum*. Outlines with camera lucida, details diagrammatic. Magnification *ca.* $\times 330$. *A*, 24-mm. larva; *B*, 58-mm. larva; *C*, 73-mm. larva; *D*, 135-mm. adult. Abbreviations: *D.*, dermis;

D.M., dermal melanophore; *D.U.C.*, dermal unpigmented cell; *E.*, epidermis; *E.C.*, epidermal cell; *L.C.*, Leydig cell; *M.*, melanophore; *S.G.*, skin gland; *S.M.*, subdermal melanophores; *S.U.C.*, subdermal unpigmented cell.

PLATE II

A, cross-section of *T. torosus* embryo, stage 44. The melanophores (*M.*) form a network, sometimes more conspicuous segmentally, over the dorsal margins of the myotomes. Grossly, they appear to form two longitudinal bands because of the deeper position of the melanophores located in the mid-line. *Ca.* $\times 80$.

B, section of skin of adult *T. torosus*, showing the large amount of melanin in the epidermal cells and the numerous epidermal melanophores, as compared to the species of *Ambystoma*, and the highly contracted dermal melanophores. *Ca.* $\times 295$.

C, section of skin of adult *A. maculatum* with grafted *T. torosus* neural crest. Section includes some donor epidermis and shows the large amount of pigment in these cells. The host epidermis is entirely lacking in pigmentation. Dermal pigmentation is distinctly reduced from that found in the normal *A. maculatum*. *Ca.* $\times 295$.

D, section of skin of adult *T. torosus* with

grafted *A. maculatum* neural crest. Section includes some donor epidermis which contains a small amount of pigment, but much less than is found in the normal *T. torosus* epidermis. Dermal pigmentation is greatly increased over that found in the normal *T. torosus*, and the melanophores are highly expanded. *Ca.* $\times 295$.

E, section of skin of an advanced larva of *T. torosus*. Epidermal pigmentation has just become heavy at this stage, and epidermal melanophores are highly expanded. Their processes extend for long distances between the epidermal cells and send off branches which enter the epidermal cells. *Ca.* $\times 295$.

Abbreviations: *D.*, dermis; *D.M.*, dermal melanophore; *D.U.C.*, dermal unpigmented cell; *E.*, epidermis; *E.M.*, epidermal melanophore; *G.E.*, graft epidermis; *H.E.*, host epidermis; *M.*, melanophore; *S.G.*, skin gland; *S.U.C.*, subdermal unpigmented cell. Outlines drawn with camera lucida, details diagrammatic.

PLATE I

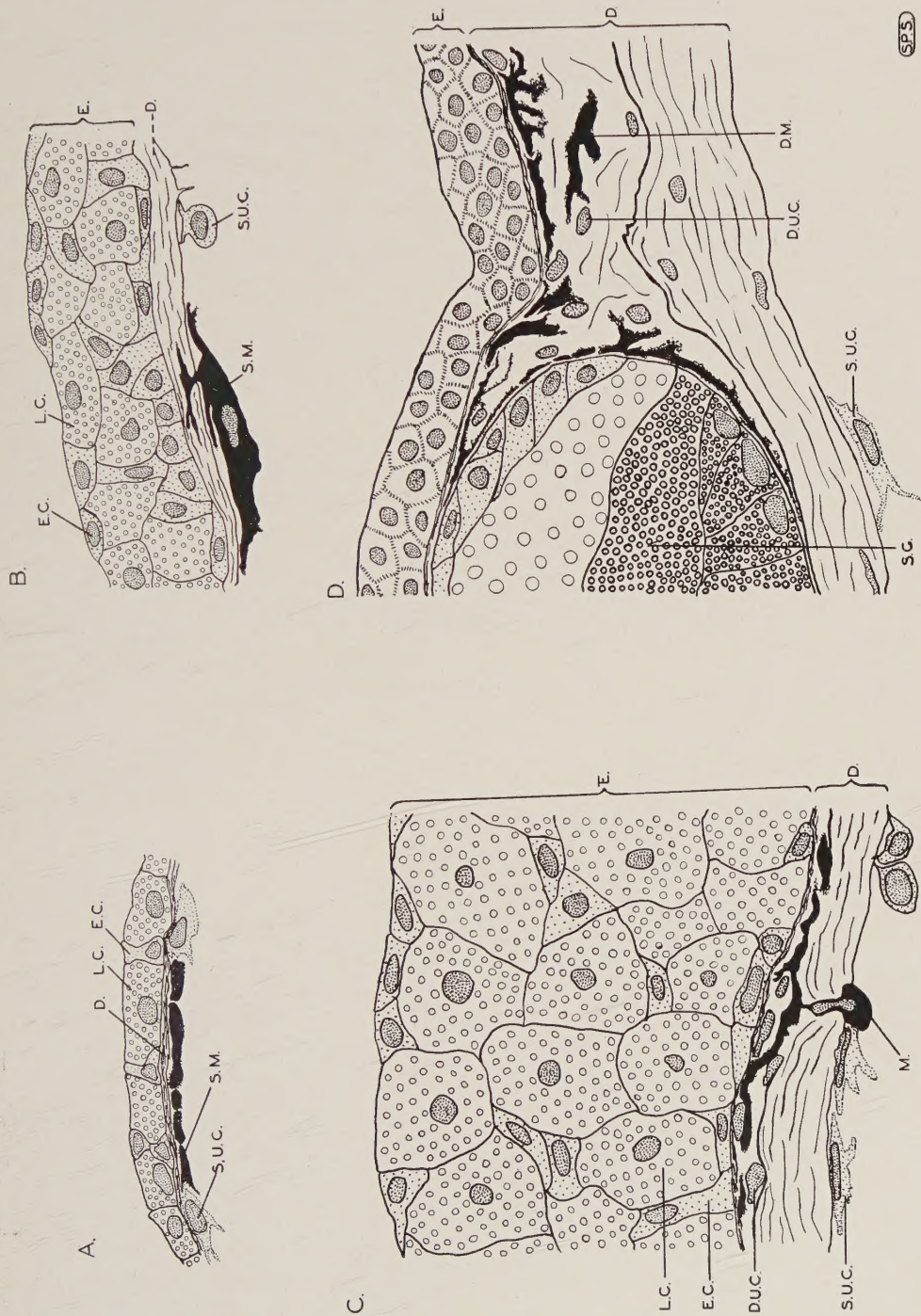
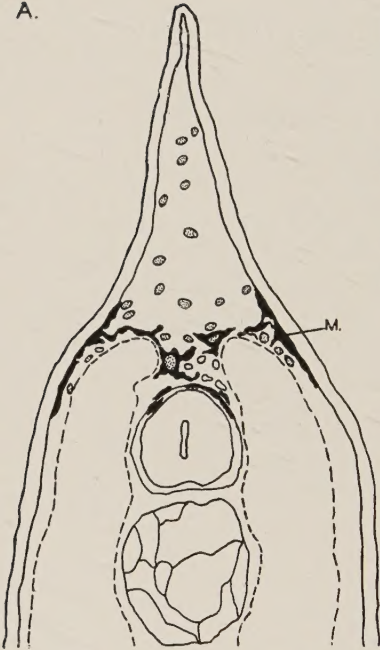
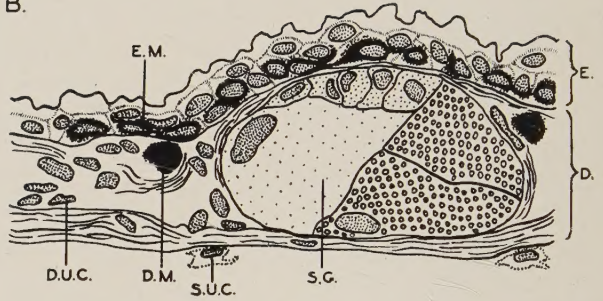


PLATE II

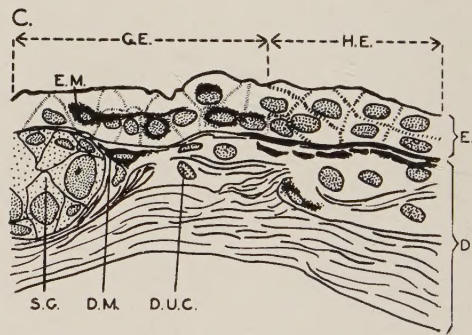
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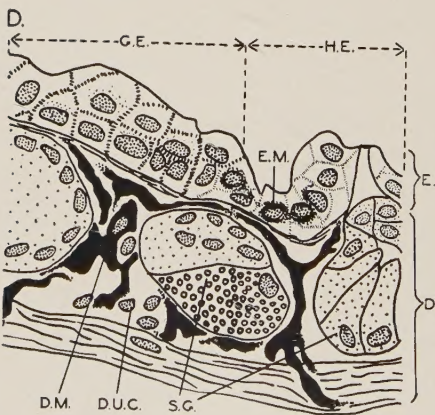
B.



C.



D.



E.

